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Intra- and inter-specific transplantation of spermatogonial stem cells in freshwater fish

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1 INTRODUCTION

Preservation of genetic resources of living organisms and biodiversity itself is one of the most important tasks of conservation biology and biology in general. Today, many species face a rapid decline in their population numbers that can lead to extinction. Major influencers that cause population loss in freshwaters are water pollution, habitat degradation, flow modifications, species invasions and overexploitation. Most of these include changes to the habitat itself, which makes conservation efforts exceptionally complex. In order to mitigate these problems, *in situ* conservation efforts such as declarations of Special Nature Reserves or ecosystem recovery programs are conducted, however, they are very limited and specific for a certain habitat. When *in situ* conservation efforts fail, different *ex situ* conservation efforts are imposed.

Ex situ conservation efforts include rearing of broodstock, creation of gene banks, seed vaults or other resource centers. Cryopreservation and cryobanking (banking of germplasm specifically) have a leading role in *ex situ* conservation as they enable a safe storage of genetic resources of a given species for an indefinite period of time. Most of the conservation efforts in fish have focused on cryopreservation of spermatozoa, especially since cryopreservation of fish eggs and embryos is not yet possible. However, this leads to problems in conducting certain conservation programs as females need to be available and need to be able to give gametes. As a method that can circumvent this problem, banking of germline stem cells (GSCs) has recently started to gain much attention from conservation biologists. The main advantage of this approach comes from the innate ability of GSCs to differentiate into gametes either *in vitro* or *in vivo* after transplantation into suitable recipients. As these cells remain functional after cryopreservation, and are able to give rise to gametes after successful manipulation (either *in vitro* culture or transplantation), GSC banking and manipulations quickly arose as a favorable alternative banking strategy and offer a novel approach in species conservation and population management.

The main aim of this dissertation was to develop novel SSC manipulation techniques which can be applied in broodstock and population management, species and population conservation, as well as in advanced propagation applications. The main focus was on developing the SSC transplantation technique and creating the onset of the surrogate production technology in several fish species (including zebrafish *Danio rerio*, common carp *Cyprinus carpio*, brown trout *Salmo trutta*, grayling *Thymallus thymallus*, European catfish *Silurus glanis* and African catfish *Clarias gariepinus*). This was complemented by optimization of cryopreservation protocols for the testicular tissue (and subsequently SSCs) which would aid in the preservation of valuable genetic resources as well as in synchronization of the transplantation technique itself.

2 MATERIAL AND METHODS

2.1 Testicular tissue sampling

Fish were euthanized by an overdose of 2-phenoxyethanol. In order to excise the testes, skin and underlying muscle on the belly of the fish between the pectoral fins were horizontally snipped and the skin and underlying muscle were cut along the belly until the anal fin thus exposing the internal organs. Testes were aseptically excised, sterilized in 70% EtOH (for few seconds) or 0.1% commercial bleach (for 2 min), washed in phosphate buffered saline (PBS), cleaned of large blood vessels and adjacent connective tissue and suspended in Leibovitz (L-15) medium. All tissues were kept on ice until further work (max 30 min).

2.2 Tissue dissociation and viability assessment

Testes (or testicular fragments) were dissociated in a solution of L-15 medium containing different proteolytic enzymes (2 mg/ml collagenase, 1.5 mg/ml trypsin unless otherwise specified) and 30-60 µg/ml DNase I. Most commonly, 500 µl of the dissociation solution was used for the digestion of each 50 mg of a tissue fragment (volume was increased according to the tissue fragment weight; e.g. 1 ml for 100 mg, 1.5 ml for 150 mg). Testicular fragments were then minced into small pieces and the dissociation was conducted at room temperature (RT; 24-28 °C) for 1.5 h on a shaking plate. The dissociation process was terminated by adding 10% FBS (v/v) and an equal amount of L-15. In order to obtain a single cell suspension, samples were filtered through 30 or 50 µm filters and centrifuged at 200 ×g for 10 min at RT. Supernatants were discarded and the pellets were resuspended in an appropriate volume of fresh L-15 medium supplemented with 10% FBS.

Viability of cells within the suspension was verified by the trypan blue (TB) exclusion test where dead cells were stained blue while live cells remained unstained. Cell suspensions were mixed with 0.4% solution of TB in a 1:1 ratio, incubated for at least 1 min at RT and the viability was checked in a hemocytometer under a phase-contrast microscope.

During optimization of cryopreservation protocols, final cell survival rate was assessed as the percentage of live cells isolated from cryopreserved tissue compared to the number of live cells isolated from the fresh tissue (i.e. the number of cells recovered from the cryopreserved tissue compared to the fresh one). The number of live SSCs was counted in 15 fields of a Bürker-Türk hemocytometer for each sample under a light microscope with phase contrast (Nikon Eclipse E600) at 40× magnification. Final cell survival rate was assessed as: $Viability\ (\%) = (N_{cryopreserved} / N_{fresh}) \times CF \times 100$ while correcting for the tissue size with a correction factor: $CF = Weight_{fresh\ tissue} / Weight_{cryopreserved\ tissue}$.

2.3 Cryopreservation

2.3.1 Freezing

Optimization of the freezing (~ 1 °C/min) protocols was conducted in sequential experiments where in each experiment one cryopreservation parameter was changed, and the best outcome was used in the subsequent experiment. For the optimization of the freezing procedure, various cryoprotectants, their concentrations, cooling rates, equilibration times, sugar and protein supplementation have been tested. However, the freezing procedure itself was similar in all experiments. Whole testes (in the case of zebrafish) or testicular fragments (in the case of other species) were loaded into 1.8 ml cryotubes filled with 1 ml of cryomedium (containing both extender and cryoprotectants). Samples were then equilibrated for a set duration on ice (most commonly between 15 and 30 min, depending on the size of the tissue) and subsequently placed into CoolCell freezing containers and placed into a deep freezer (- 80 °C) enabling the cooling rates of ~ 1 °C/min. When samples reached approximately -80 °C after 1.5 h in the deep freezer, they were plunged into the liquid nitrogen and stored in a storage dewar. Alternatively, samples were frozen using a Controlled rate freezer (IceCube 14S programmable freezer) with different cooling rates down to - 80 °C before being plunged into liquid nitrogen. After at least one day of storage, cryotubes were thawed in a 25 °C water bath (10 °C in the case of trouts) for at least 2 min and the testes were rehydrated in three changes of L-15.

2.3.2 Vitrification

Vitrification of testes was conducted by needle-immersed vitrification (NIV) method. Namely, whole testes (zebrafish) or testicular fragments (other species) were pinned to an acupuncture needle and incubated in an equilibration solution (ES) and subsequently in a vitrification solution (VS) for a certain time period (specific for each species). Excess liquid was carefully absorbed from the tissue by a sterile paper towel and the needles were plunged in liquid nitrogen. Needles were then placed into 5 ml cryotubes and stored in a storage dewar. After at least one day of storage, tissues were warmed in three sequential warming solutions (WS) at room temperature (RT) for different periods of time. All warming solutions contained L-15 supplemented with 10% FBS and various concentrations of sucrose.

2.4 Transplantation

All transplantations were conducted into larvae of the recipient species. Recipient larvae were anesthetized in 0.03% 2-phenoxyethanol and transferred into a petri dish coated with 2% agar. Fresh and/or cryopreserved SSCs were then injected into the abdominal cavity of each recipient larvae. In cyprinids, the tip of the needle was inserted into the body cavity either (1) between the swimming bladder and the intestines, or (2) behind the swimming

bladder. In salmonids, the two entry points were (1) between the dorsal artery and the gut, or (2) just above the yolk sac. Visualization of cells flowing within the body cavity and around intestines (or yolk sac) was a sign of a successful injection/transplantation. After transplantation, recipient larvae were kept in system water, and one day after transplantation they were transferred into aquaria and fed with *Artemia* nauplii and/or artificial food. Control groups of intact fish and morphants were exposed to the same rearing conditions as the experimental individuals were; however, no operations were conducted on them. In order to verify transplantation efficiency, recipient fish were dissected several months after transplantation (depending on the species), and their gonads were checked for a fluorescent signal. Fish were euthanized in an overdose of 2-phenoxyethanol and dissected by removing the head and tail and placed dorsoventrally under an epifluorescent microscope (Nikon Eclipse 600) or a Leica M205FA stereomicroscope. Detection of one or more fluorescent cells within the gonadal tissue was evidence of successful colonization of the recipient gonads by the transplanted cells.

3 RESULTS AND DISCUSSION

3.1 Allogenic transplantation of fresh and cryopreserved SSCs produces viable donor-derived offspring in zebrafish

3.1.1 Isolation of SSCs

Dissociation media tested in this study had a significant effect on the yield of SSCs where the highest yield was observed when using media containing either 3 mg/ml trypsin + 50 µg/ml DNase I or 2 mg/ml collagenase + 1.5 mg/ml trypsin + 50 µg/ml DNase I. The utilization of collagenase as the sole proteolytic enzyme left many undissociated cell clumps, while the supplementation of the unspecific enzyme such as trypsin cleaved the additional cell bonds and produced a monodisperced cell suspension.

3.1.2 Optimization of the freezing and vitrification protocols

The viability of spermatogonia frozen with the addition of 1.3 M Me_2SO in the cryomedium was significantly higher than the viability of those frozen with other tested cryoprotectants in the same concentration (Fig. 1A). When testing the effects of different Me_2SO concentrations, viability was the highest when cryopreserving with 1.3 M (Fig. 1B). The supplementation of cryomedium containing 1.3 M Me_2SO with different sugars (glucose, sucrose, fructose and trehalose in 0.1 and 0.3 M) did not yield significant differences among the tested groups (Fig. 1C), therefore 0.1 M trehalose was used in further trials. Finally, the addition of different protein fractions as non-permeating cryoprotectants (1.5% BSA, 1.5% FBS, 1.5% skim milk and 10% egg yolk) was assessed. Only the presence of 1.5% skim milk produced significantly lower germ cell viability (Fig. 1D). Therefore, cryomedium containing 35.2%

extender, 1.3 M Me_2SO, 0.1 M trehalose and 1.5% BSA was used in transplantation trials.

Only the vitrification solutions had a significant effect on the testicular germ cell viability after warming. The highest viability was obtained when combining VS3 containing lower concentrations of PG and Me_2SO (3 M of both) with either ES1 (containing 1.5 M MeOH and 1.5 M PG; 48.04±11.45%) or ES3 (containing 1.5 M PG and 1.5 M Me_2SO; 40.69±29.9%) (Fig. 1F). As high concentrations of cryoprotectants (usually above 5 M) can be toxic to cells, the utilization of equal and thus smaller concentrations of two cryoprotectants can have a beneficial effect where enough cryoprotectant is present, however, not one of them is in a high enough concentration to cause toxic effects to the cells. The combination of ES1 and VS3 was used in transplantation trials.

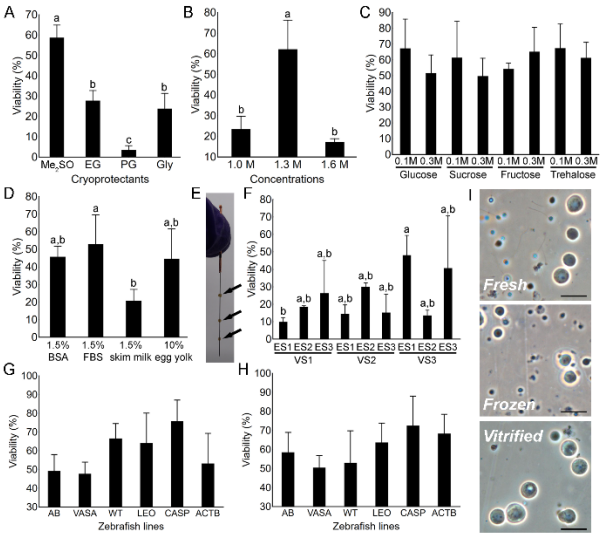

Fig. 1. Optimization of the slow-rate freezing (A-D) and vitrification (F) protocols in zebrafish. (**A**) Viability of spermatogonia after freezing with 1.3 M dimethyl sulfoxide (Me_2SO), ethylene glycol (EG), propylene glycol (PG) and glycerol (Gly). (**B**) Viability of spermatogonia after slow-rate freezing with 1.0, 1.3 and 1.6 M of Me_2SO. The effects of sugar (**C**) and protein (**D**) supplementation of spermatogonia viability. (**E**) Testes (arrows) pinned on an acupuncture needle for the needle-immersed vitrification (NIV) method. (**F**) The effects of different equilibration (ES) and vitrification (VS) solutions on spermatogonia viability after NIV. Efficiency of the developed freezing (**G**) and vitrification protocols (**H**) demonstrated on AB wild type (AB), *vasa::egfp* (*ddx4*$^{sa6158/sa6158}$) (VASA), *Wilms tumor::egfp* (*wt1b*) (WT), leopard (*gja5b*^{t1}) (LEO), casper (*mitfa*$^{w2/w2}$*; mpv17*$^{a9/a9}$) (CASP) and *β-actin:yfp* (*pku341Tg*) (ACTB) zebrafish lines. (**I**) Testicular cell suspensions prior to, and after cryopreservation.

3.1.3 Efficiency of the cryopreservation protocols in different zebrafish transgenic lines

Efficiency of the optimal freezing and vitrification protocols was tested by cryopreserving whole testes of six different zebrafish lines (AB wild-

type, casper (*mitfa*$^{w2/w2;}$ *mpv17*$^{a9/a9}$), leopard (*gja5b*^{t1}), *vasa::egfp* (*ddx4*$^{sa6158/sa6158}$) transgenic line, *Wilms tumor::egfp* (*wt1b*) transgenic line and *β-actin:yfp* (*pku341Tg*) transgenic line). Both freezing and vitrification protocols proved to be efficient and reproducible since they yielded viability rates of nearly (or higher than) 50% (Fig. 1G, 1H and 1I).

3.1.4 Transplantation of cryopreserved SSCs and production of donor-derived progeny

To determine whether spermatogonia are functional and still have the capacity to colonize the recipient gonads and proliferate inside them after cryopreservation, fresh, frozen/thawed and vitrified/warmed spermatogonia from *vasa::egfp* transgenic line were transplanted into wild AB type zebrafish larvae (7 dpf). Recipients from all three groups dissected 50 days after transplantation displayed green fluorescent signal within their gonads indicating that donor cells had the ability to colonize the recipient gonads (Fig. 2A-C). The number of recipients containing incorporated donor-derived spermatogonia did not differ among the groups and was around 25% in all groups.

After the initial transplantation assay, spermatogonia from *actb:yfp* transgenic line were transplanted into sterilized (by *dnd*-morpholino oligomer; *dnd*-MO) wild-type AB larvae (7 dpf). Upon reaching maturity, all recipient individuals morphologically appeared to be male. Dissection (six months after transplantation) of the MO-injected control individuals revealed no signs of germline development as the gonads were comprised of somatic cells only (Fig. 2D-D1). Dissection of recipient fish revealed that all developing gonads displayed green fluorescent signal corroborating the previously observed results that fresh, frozen/thawed and vitrified/warmed spermatogonia retain their ability to colonize and proliferate within recipient gonads (Fig. 2E-E1). Expression of *yfp* was further confirmed by RT-PCR using RNA extracted from the resulting fluorescent testes. Additionally, recipients of all three groups produced milt. Both obtained milt (Fig. 2F) and individual spermatozoa (Fig. 2F') displayed a green fluorescent signal, which was additionally corroborated with positive RT-PCR amplification of *yfp* (Fig. 2G). Milt volume (Fig. 2H) and sperm counts (Fig. 2I) did not significantly differ between the recipient fish and AB wilt type and *actb:yfp* control individuals. None of the sterilized control individuals produced any milt.

Upon reaching maturity (six months after transplantation), recipient fish were naturally mated with wild type AB females to produce progeny. Fertilization and hatching rates were similar between all tested groups (Fig. 2J). All produced larvae displayed green fluorescent signal similar to that of the *actb:yfp* larvae indicating that the larvae were of donor-derived origin (Fig. 2K). The expression of *yfp* in larvae obtained from mated recipient fish was

additionally confirmed through RT-PCR amplification using total RNA extracted from the resulting larvae (Fig. 2L).

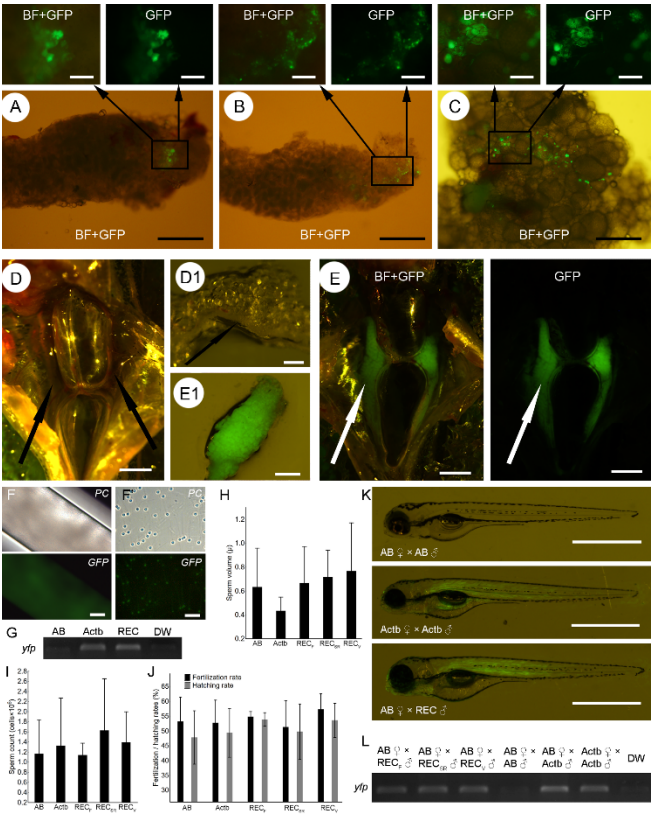

Fig. 2. Incorporation of fresh and cryopreserved spermatogonia and production of donor-derived progeny in zebrafish. Incorporation and proliferation of fresh (**A**) and cryopreserved (**B, C**) *vasa::egfp* spermatogonia within the testes (**A, B**) and ovaries (**C**) of non-sterilized recipients. Testes (arrows) of the control MO-sterilized recipients (**D**) appear undeveloped (**D1**), while recipients of *β-actin:yfp* spermatogonia displayed developed testes (arrows) (**E**) with strong green fluorescence (**E1**). Milt (**F**) and individual spermatozoa (**F'**) stripped from recipients demonstrating yfp fluorescence. (**G**) RT-PCR amplification of *yfp* in milt obtained from wild type AB (AB), *β-actin:yfp* transgenic (Actb) and recipient (REC) zebrafish. Milt volume (**H**) and sperm count (**I**) of milt obtained from wild type AB (AB), *β-actin:yfp* transgenic fish (Actb) and recipients of fresh (REC_F), frozen (REC_{SR}) and vitrified (REC_V) spermatogonia. (**J**) Fertilization rates after spawning control fish as well as recipient males and wild-type AB females. (**K**) Three dpf larvae obtained from crossing control AB males and recipient males (REC) with wild-type AB females, as well as larvae obtained from crossing Actb males and females under fluorescent stereomicroscope. (**L**) PCR amplification of *yfp* in offspring obtained from control crossings, as well as from crossing recipient males with wild-type AB females.

Current study displayed that spawning of the wild-type recipient fish with wild-type females produced viable offspring demonstrating donor-derived

traits. An important issue to factor in is that the offspring will be heterozygous for the assessed trait. Therefore, pure homozygous individuals can be obtained only in the F2 generation, where approximately 25% of the F2 offspring will be homozygous for the given trait. An alternative way to obtain 100% homozygous individuals in the F1 generation is to transplant SSCs into non-sterilized recipients, and cross sterilized recipient males with non-recipient sterilized females where a percentage of progeny will be 100% homozygous, or to feminize sterilized recipients with 17α-estradiol (EE2), in which case (if successful), spawning of sterilized recipients will create 100% homozygous progeny.

3.2 Cryopreservation and transplantation of common carp SSCs

3.2.1 Hypothermic storage

Two hypothermic storage (at 4 °C) trials were conducted: (1) firstly to determine the optimal sample type for the hypothermic storage (storing tissue pieces vs isolated cells within a suspension) and (2) to determine the viability of SSCs during two weeks of storage within a cell suspension. During the first 24-h-long storage trial, SSCs stored in cell suspensions showed higher viability compared to the tissue pieces. Viability of cells in cell suspensions remained similar to the fresh control. In tissue pieces, SSCs preserved in L-15 retained their viability during the first six hours while SSCs preserved in DMEM displayed significantly lower viability results compared to the fresh control. During the two-week preservation study conducted only on cell suspensions, viability of SSCs stored in L-15 significantly decreased after 1.5 days, however a plateau of approximately 80–70% viability remained until the 10th day. On the other hand, the viability of SSCs stored in DMEM decreased steeply during the first three days, after which a plateau of approximately 60–45% remained until the end of the experiment. L-15 yielded slightly higher viability than DMEM during the storage time, however clear statistical delineations were not observed.

3.2.2 Freezing and vitrification of common carp testicular tissue

Among all tested cryoprotectants, the highest viability was observed using Me_2SO (8.4%) (Fig. 3A). Combination of different Me_2SO concentrations (1 to 3 M) and freezing rates (-0.5 to -10 °C/min) resulted in a wide range of viability among different combinations. Viability over 20% was recorded only when combining a -1 °C/min freezing rate with 2 M and 2.5 M Me_2SO (Fig. 3B). Generally, slower cooling rates (-0.5 to -2.5 °C/min) resulted in higher viability in comparison to the faster cooling rates (-5 to -10 °C/min), while the resistance to the fastest cooling rate increased with higher Me_2SO concentration. Additionally, the use of higher Me_2SO concentrations and faster cooling rates resulted in higher amount of viable spermatozoa in cell

suspensions indicating that optimal conditions for spermatozoa and spermatogonia are different.

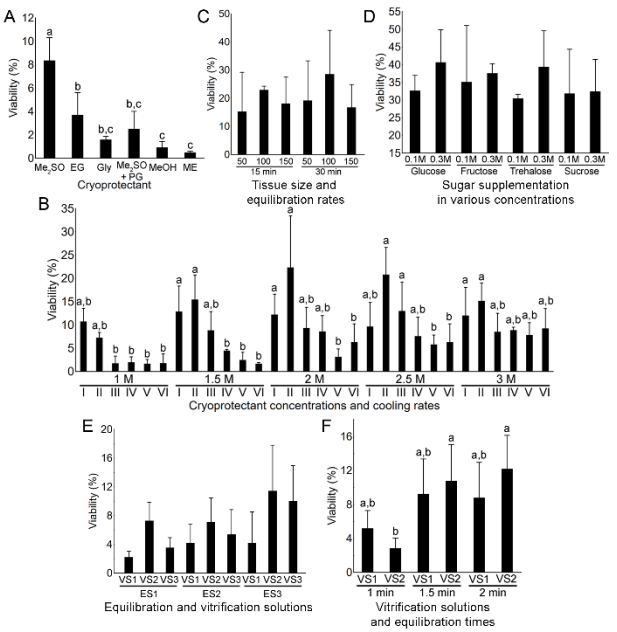

Fig. 3. Optimization of the freezing (A-D) and vitrification (E, F) protocols for common carp spermatogonia. (**A**) Viability of spermatogonia after freezing with 1.5 M Me_2SO, EG, Gly, Me2SO+PG, MeOH and ME. (**B**) The effects of Me_2SO concentrations (1, 1.5, 2, 2.5 and 3 M) and cooling rates of 0.5 (I), 1 (II), 2.5 (III), 5 (IV), 7.5 (V) and 10 (VI) °C/min on spermatogonia viability. (**C**) Viability of spermatogonia after exposing 50, 100 or 150 mg tissue fragments for 15 or 30 min to the cryomedium. (**D**) Effect of sugar supplementation of spermatogonia viability. Effects of different equilibration (ES) and vitrification (VS) solutions (**E**) and exposures (1, 1.5 and 2 min) to different VS (**F**) on spermatogonia viability after NIV.

Exposure of tissue pieces of different sizes (50 – 150 mg) to cryoprotectants for variable periods of time (15 or 30 min) did not result in high variability. The highest viability was attained when equilibrating 100-mg tissue pieces for 30 min, however, statistical differences were not significant in comparison to other combinations (Fig. 3C). Lastly, the supplementation of cryomedia with various sugars (glucose, fructose, trehalose and sucrose) in different concentrations (0.1 or 0.3 M) did not result in significant differences (Fig. 3D). The highest viability of ~ 40% was obtained when equilibrating 100 mg tissue pieces for 30 min in a cryomedium containing 2 M Me_2SO, 0.3 M glucose, 1.5% BSA and 25 mM HEPES.

In the first vitrification trial, only the VSs displayed a significant effect on the viability of spermatogonia after warming. Even though the average viability was higher when combining ES3 (containing 1.5 M PG and 1.5 M Me_2SO) with either VS2 (containing 1.5M MeOH and 5.5 M Me_2SO) or VS3

(containing 3 M PG and 3 M Me_2SO), clear statistical differences could not be observed (Fig. 3E). Therefore, VS2 and VS3 were used in the subsequent experiment. In the second trial, exposure times to the vitrification solutions had a significant effect on spermatogonia viability. Only exposure for 1 min to VS2 (containing 3 M PG and 3 M Me_2SO) yielded significantly lower viability rates compared to other groups (Fig. 3F).

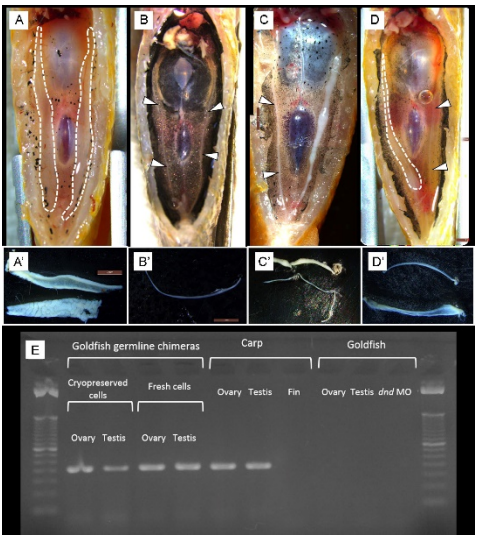

Fig. 4. Detection of common carp spermatogonia incorporation and proliferation after interspecific transplantation into sterilized goldfish recipients. (**A-D**) Ventral view of dissected goldfish recipients. (**A'-D'**) Stereomicroscopic observation of the dissected gonads. (**A, A'**) Control fish displaying both gonads fully developed. (**B, B'**) dnd-MO treated goldfish displaying a lack of gonadal development. Development of testis (**C, C'**) and ovary (**D, D'**) after transplantation of common carp spermatogonia into dnd-MO sterilized goldfish recipients. Developed gonads are outlined with white dashed lines, undeveloped gonads are pointed out by white arrowheads. (**E**) RT-PCR amplification of common carp *dnd* amplicon in gonads of goldfish germline chimeras, as well as in control common carp gonads and fin and goldfish gonads.

3.2.3 Transplantation of cryopreserved SSCs

Due to the higher overall viability obtained by freezing (40.7 ± 9.2%) compared to vitrification (11.4 ± 4.9%), only spermatogonia frozen with the optimized protocol were transplanted alongside freshly isolated cells into the recipient goldfish larvae. Recipient embryos were sterilized by injecting *dnd*-MO, and the success of sterilization was confirmed by fluorescent microscopy after co-injection with GFP-nos1 3′UTR mRNA. All of the co-injected larvae displayed a successful depletion of recipient's endogenous PGCs.

Success of transplantation was assessed three months after transplantation where the recipients were visually inspected for developing gonads after dissection, as well was by RT-PCR amplification of carp-specific *dnd* amplicons. During the visual inspection, all of the non-treated controls displayed normally developing gonads (Fig. 4A, 4A'), while none of the MO-treated control individuals showed any signs of developing gonads (Fig. 4B, 4B'). Approximately 40% of the recipients injected with frozen/thawed carp

spermatogonia displayed developing gonads, while ~ 50% of recipients injected with fresh spermatogonia displayed developing gonads. Developing gonads were either testes characterized by their while color (Fig. 4C, 4C') or ovaries distinguishable by the presence of oocytes observable under higher magnification (Fig. 4D, 4D'). Donor-derived origin of the germ cells within the developing recipient gonads was determined by RT-PCR amplification of the carp *dnd* amplicon (Fig. 4E). These results indicated that both fresh and frozen/thawed carp spermatogonia successfully migrated and incorporated into the goldfish gonads, as well as proliferated within the recipient gonads and produced later-stage germ cells of both sex.

3.3 SSC cryopreservation and interspecific transplantation as a new light in the conservation of valuable Balkan trout genetic resources

3.3.1 Isolation of brown trout SSCs

The highest total yield was observed in the two groups without trypsin (2 mg/ml and 6 mg/ml collagenase). As the protocol using 6 mg/ml collagenase displayed significantly lower viability, protocol using 2 mg/ml collagenase displayed the highest efficiency, i.e. it yielded the highest number of viable cells and was used in all subsequent tissue dissociations.

3.3.2 Germ cell labelling and transplantation

In order to determine the optimal volume of PKH-26 dye needed for labelling of donor germ cells prior to transplantation, 1, 2 and 3 µl of dye were tested for labelling of 1 million cells. The highest dye volume (3 µl) was considered to be optimal since the fluorescent signal was strong and 90% of the cells were labelled, therefore this volume of dye was used for the staining of the germ cell suspensions used in transplantations.

SSCs of brown trout and grayling were transplanted into larvae of rainbow trout. Recipient larvae were reared until 60 days post-transplantation at which point the average survival rate was 59.5±7.6%. After dissection, fluorescently labelled cells could be detected within the recipient gonads (Fig. 5A). Fluorescent signal was of similar intensity in all recipients without regard to cells or donors. This indicated that SSCs of both donor species could migrate within the abdominal cavity of the rainbow trout recipients and colonize their gonads. Control individuals displayed no fluorescence after dissection.

Additionally, PCR amplification of the brown trout/grayling mtDNA CR using DNA extracted from recipient gonads further corroborated the results obtained by fluorescent microscopy. On average, 71% (6/7 BT and 3/4 G) of individuals displaying fluorescent signal displayed positive amplification of brown trout / grayling – specific fragments (Fig. 5B). Furthermore, individuals negative for the fluorescent signal did not result in any brown trout / grayling specific amplification (Fig. 5B). The results of this PCR analysis were

qualitative and did not give any quantitative indication on the amount of donor mtDNA nor the number of donor cells within the recipient gonads.

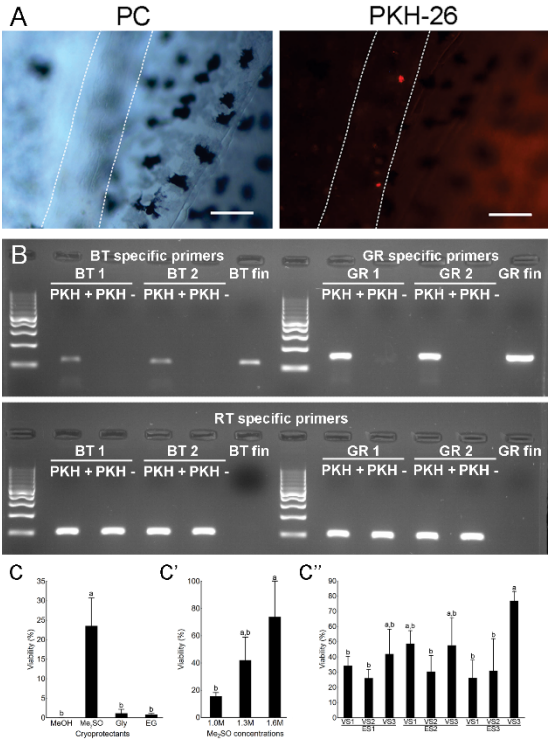

Fig 5. Verification of transplantation success of brown trout and grayling SSCs into rainbow trout recipients and cryopreservation of brown trout SSCs. (**A**) Detection of the fluorescently labelled germ cells within the recipient gonads (delineated by white lines) signified successful incorporation of the donor-derived germ cells. Scale bars: 100 μm. (**B**) Polymerase chain reaction (PCR) amplification of the mtDNA CR of brown trout (BT; upper panel), grayling (GR; upper panel) and rainbow trout (RT; lower panel) from the rainbow trout recipient gonads (60 dpt). DNA isolated from two recipients displaying positive PKH-26 fluorescent signal (PKH+), two recipients not displaying any signal (PKH-) and brown trout and grayling fin tissue was used as a template. (**C**) Viability of SSCs after freezing with 1.3 M MeOH, Me_2SO, Gly and EG. (**C'**) Viability of SSCs after freezing with either 1.0, 1.3 or 1.6 M Me_2SO. (**C''**) The effects of different equilibrations solutions (ES1 – ES3) and vitrification solutions (VS1 – VS3) of SSCs after NIV.

3.3.3 Cryopreservation of brown trout testicular tissue

The highest viability in the first trial was obtained by using Me_2SO (Fig. 5C). When testing different Me_2SO concentration, the highest viability (~75%) was obtained after using 1.6 M (Fig. 5C'). To test the functionality of the frozen cells, cryopreserved SSCs were transplanted into rainbow trout larvae. Six months after transplantation, PCR amplification of the brown trout mtDNA CR using DNA extracted from recipient gonads demonstrated that 4 of 23 analyzed recipients (17%) displayed a positive signal. As this percentage is similar to the colonization rates of fresh SSCs (19% obtained after PCR amplification), we have demonstrated that the frozen cells do retain their ability to colonize recipient testes after transplantation.

The vitrification protocol was optimized by testing three equilibration and three vitrification solutions similarly to trials in other fish species. The highest viability was obtained when combining ES3 and VS3 (which contains equal concentrations of PG and Me_2SO) (Fig. 5C'').

3.4 Cryopreservation and in vitro culture of catfish early-stage germ cells

3.4.1 Cryopreservation of catfish testicular tissue

In both European and African catfish, cryoprotectants and their concentrations had a significant effect on post-thaw viability, while the extenders did not have a significant effect. In European catfish, the use of 3 M Me_2SO with either PBS or Yoshizaki extender yielded the highest SSC viability (Fig. 6A). In African catfish on the other hand, the highest SSC viability was observed when using 3 M Me_2SO with Yoshizaki extender and 3 M EG with PBS (Fig. 6B). Therefore, cryomedium containing 35.2% Yoshizaki extender and 3 M Me_2SO was determined as the optimal for both catfish species.

During the vitrification trial, no significant differences were obtained among the experimental groups in either of the tested species (Fig. 6C, 6D). As the highest average post-warming viability in both species was below 20%, freezing of catfish testicular pieces was evidently superior to vitrification.

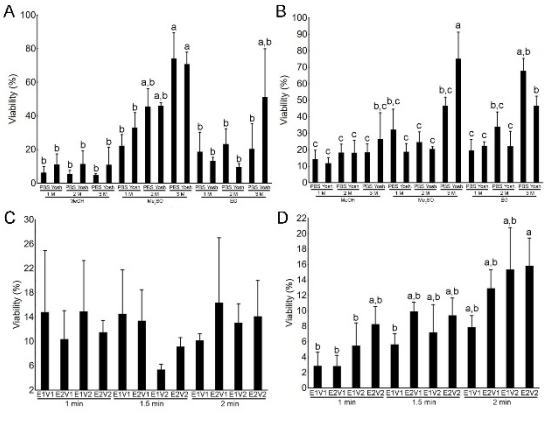

Fig. 6. Optimization of the cryopreservation protocols of European and African catfish SSCs. Viability of European catfish (**A**) and African catfish (**B**) SSCs after freezing with 1, 2 or 3 M of MeOH, Me_2SO or EG in either PBS or Yoshizaki (Yosh) extender. The effect of different ES, VS and exposure times to VS on the viability of European catfish (**C**) and African catfish (**D**) SSCs.

3.4.2 In vitro culture of catfish early-stage germ cells

To test the functionality of frozen catfish early-stage germ cells, Ficoll enriched cell fraction obtained after freezing African catfish testicular tissue was seeded into an *in vitro* cell culture. Seeded cells were grown in a suspension culture using a testicular culture medium supplemented with various hormones and growth factors. Individual cells that were seeded at the start of the culture started to form small spherical aggregates of approximately 15-20 cells during

the first day (Fig. 7). As the culture period progressed, the size of aggregates became larger which was indicative of cell proliferation.

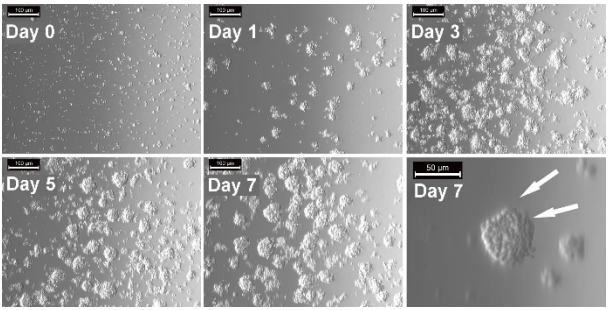

Fig. 7. *In vitro* spermatogenesis of African catfish testicular cells in a suspension culture. By day 7, tails of spermatozoa start to appear on the edges of the aggregates (arrows).

4 CONCLUSIONS AND NEW SCIENTIFIC RESULTS

During the course of this thesis, key aspects of spermatogonial stem cell manipulation have been described in several fish species. These aspects include isolation of SSCs, development of optimal protocols for both short- and long-term storage of these cells, as well as their transplantation through which surrogate production can be initiated and *in vitro* culture. Species-specific conclusions obtained in the thesis are the following:

- Reproducible slow-rate freezing and vitrification protocols have been developed for zebrafish SSCs. Through SSC transplantation technique, donor-derived offspring can be obtained from surrogate parents.
- Freezing and vitrification protocols have been developed for common carp SSCs. SSCs are functional after thawing, and have the ability to incorporate, proliferate and differentiate into spermatozoa and early-stage oocytes in goldfish recipients after transplantation.
- Brown trout and grayling SSCs have the ability to colonize and start proliferating within recipient rainbow trout gonads. Combined with the cryopreservation protocols developed, this technique offers new valuable opportunities for the conservation of valuable Balkan trout species and populations.
- Cryopreservation protocols for two catfish species (European and African catfish) have been developed. With the utilization of *in vitro* culture and spermatogenesis developed in this study, new and advanced fertilization strategies can be implemented during propagation of these species.